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A hundred-year-old question: is the moss calyptra covered by a cuticle? A case study of *Funaria hygrometrica*

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• *Background and Aims* The maternal gametophytic calyptra is critical for moss sporophyte development and ultimately sporogenesis. The calyptra has been predicted to protect the sporophyte apex, including the undifferentiated sporogenous region and seta meristem, from desiccation. We investigate the hypothesis that this waterproofing ability is due to a waxy cuticle. The idea that moss calyptrae are covered by a cuticle has been present in the literature for over a century, but, until now, neither the presence nor the absence of a cuticle has been documented for any calyptra.
• *Methods* The epidermis of the calyptra, leafy gametophyte and sporophyte sporangia of the moss *Funaria hygrometrica* were examined using scanning and transmission electron microscopy. Thicknesses of individual cuticle layers were quantified and compared statistically. The immunochemistry antibody (LM19) specific for pectins was used to locate cell wall material within the cuticle.
• *Key Results* A multi-layered cuticle is present on the calyptra of *F. hygrometrica*, including layers analogous to the cuticular layer, cell wall projections, electron-lucent and electron-dense cuticle proper observed in vascular plants. The calyptra rostrum has a cuticle that is significantly thicker than the other tissues examined and differs by specialized thickenings of the cuticular layer (cuticular pegs) at the regions of the anticlinal cell walls. This is the first documentation of cuticular pegs in a moss.
• *Conclusions* The calyptra and its associated cuticle represent a unique form of maternal care in embryophytes. This organ has the potential to play a critical role in preventing desiccation of immature sporophytes and thereby may have been essential for the evolution of the moss sporophyte.

Key words: Bryophyte, calyptra, cuticle, cuticular pegs, desiccation, electron microscopy, *Funaria hygrometrica*, maternal care.

INTRODUCTION

The evolution of maternal care was a critical innovation for the successful colonization of the land by plants (Graham *et al.*, 2000). In mosses, embryos begin development surrounded by the maternal gametophyte tissues of the archegonium and subtending gametophyte (Crum, 2001). Ultimately this encapsulating tissue separates into a ring surrounding the sporophyte base and the calyptra, a cap covering the sporophyte apex that is thereafter disconnected from the maternal gametophyte (Janzen, 1917). Over a century ago, Hy (1884) hypothesized that moss calyptrae are covered by a cuticle that protects the developing sporophyte apex from desiccation. Sporophytes with their calyptra removed only survive when grown in a high-humidity chamber (Bopp, 1957; French and Paolillo, 1975*a*), indicating a role in desiccation prevention. Bopp and Stehle (1957) demonstrated that a dye solution ascended sporophytes faster when they lacked calyptrae, suggesting that the calyptra prevents water loss from the apex. Despite the ongoing reiteration of this hypothesis (Janzen, 1917; Schofield and Hébant, 1984), neither the presence nor the absence of a cuticle has been documented conclusively for any moss calyptra.

A cuticle occurs on other bryophyte organs, including sporophytes and thalloid gametophytes (Neinhuis and Jetter, 1995; Cook and Graham, 1998). Bryophyte cuticles are chemically similar but less complex than the cuticles of vascular plants and often consist of a thin procuticle, with few taxa developing cuticular layering (Sack and Paolillo, 1983; Cook and Graham, 1998). Furthermore, surface studies of bryophytes using scanning electron microscopy (SEM) show epicuticular wax ornamentations similar to those of vascular plants (Proctor, 1979; Koch *et al.*, 2009).

Calyptra removal experiments demonstrated that this organ influences moss sporophyte development and is critical for both sporangium and spore formation (Herzfelder, 1923; Bopp, 1954; French and Paolillo, 1975*a*). Two previously examined hypotheses relate to hormonal secretion (physiological) and mechanical pressure (physical) from the calyptra (Bopp, 1984). Calyptrae were treated to remove physiologically active compounds and replaced on the apices of developing sporophytes. Covered by these inactive calyptrae, sporophytes successfully completed their development, including sporogenesis (Bopp, 1957; French and Paolillo, 1975*a*), indicating that physiologically active compounds, if present, are not required for sporophyte development. Conversely, sporophytes without calyptra failed to complete sporogenesis, demonstrating that the physical presence of the calyptra is necessary, thus supporting the mechanical pressure hypothesis.

As a maternal gametophyte tissue that maintains prolonged contact with its sporophyte offspring, the calyptra represents

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an understudied example of maternal care in mosses. Support for the hypothesis that the calyptra acts as a waterproof cap, functioning to protect the developing sporophyte apex from desiccation, would be provided by the presence of a water-loss barrier, most probably a cuticle or cuticle-like layer on the calyptra. Here we document the presence of a cuticle on the outer epidermal cells of the calyptra and compare its structure with that found in the leafy portion of the gametophyte and sporophyte sporangia.

MATERIALS AND METHODS

Study taxon

The moss *Funaria hygrometrica* Hedw. is a cosmopolitan, pioneer species found in disturbed habitats. Sporophytes range from 12 to 80 mm tall (Fig. 1A, B; Miller and Miller, 2007) with structurally complex capsules that can contain more than 100 000 wind-dispersed spores (Shaw, 1991). The calyptra of *F. hygrometrica* is 2–5 mm long, including a narrow rostrum above a wider inflated base (Fig. 1C). Plants used in the study were from a Connecticut population (CONN Budke #145) cultured in the laboratory in PlantCon plant tissue culture containers (MP Biomedicals, Solon, OH, USA) on a rich sandy loam soil mix. Gametophytes were grown from spores of the original population for at least 4 months at room temperature under 16 h of daylight. Plants were cold treated at 10 °C with 8 h daylight for 2 months to stimulate formation of antheridia and archegonia (Dietert, 1980). Fertile populations were flooded with water for 24 h and remained in the cold growth chamber for 1 week after un-flooding. They were then placed at room temperature under 16 h of daylight. Calyptra and sporophytes from this laboratory population were harvested at a late stage of sporophyte development (capsule expanded with calyptra present on the apex; Fig. 1B) and used for all subsequent experiments. Additionally, mature gametophytes with fully expanded leaves were collected from this population.

Scanning electron microscopy

To document the presence and structure of epicuticular waxes, mature samples of calyptrae, sporangia and leafy gametophytes were air dried for at least 48 h, then rinsed in two 30-s washes of either water or chloroform; the latter has been shown to remove epicuticular waxes (Proctor, 1979). Air-dried only ($n = 3$ for each tissue), water-rinsed ($n = 3$ for each tissue) and chloroform-rinsed samples ($n = 3$ for each tissue) were processed and examined using SEM according to previously published methods (Budke *et al.*, 2007).

Transmission electron microscopy (TEM)

To investigate the internal structure of the cuticle, sporangia were cut off after calyptrae removal, with both tissues split longitudinally to facilitate fixation and infiltration. All tissues (calyptrae $n = 3$, sporophytes $n = 3$, leafy gametophytes $n = 3$) were immediately placed into fixative (1·5 % glutaraldehyde, 1·5 % formaldehyde in 0·05 M PIPES buffer, pH 7·0; Renzaglia *et al.*, 1997) for 4–8 h under ambient conditions, overnight under vacuum and then for the next 5 d in the dark at 4 °C, for a total of 6 d of fixation. Tissues were rinsed in 0·05 M PIPES buffer twice for 20 min each and kept in buffer overnight at 4 °C in the dark. Tissues were rinsed in 0·05 M PIPES buffer once for 20 min. Osmium fixation (2·0 % OsO_4 in 0·05 M PIPES buffer, pH 7·0) was carried out for 2 h in the dark at 4 °C followed by three changes of distilled water for 30 min each. Dehydration was performed using a graded ethanol (EtOH) series of cold solutions with 30 min at each stage, with a two final rinses of 100 % EtOH for 15 min each. After this step, tissues were embedded in either Spurrs or LR White resin.

Spurrs resin (SR). The following solutions were mixed: 10 g ERL4206, 4·75 g DER Ro, 26 g NSA and 0·4 g DMAE (Pelco, Redding, CA, USA). The infiltration process was carried out on a rolling mixer with perforated lids for the following solutions: SR and 100 % EtOH (1 : 1) for 1·5 h, SR and 100 % EtOH (3 : 1) for 3 h, 100 % SR overnight (~13 h) and 100 % SR (~5 h). Block silicon moulds were used and resin blocks were polymerized for 9 d in an oven at 40–45 °C.

LR White resin (LRWR). LRWR (500 g) was mixed with 9·9 g of accelerator (Pelco). Infiltration was carried out on a rolling mixer with perforated lids: LRWR and 100 % EtOH (1 : 1) for 1·5 h, LRWR and 100 % EtOH (3 : 1) for 3 h, 100 % LRWR overnight (~13 h) and 100 % LRWR (~7 h).

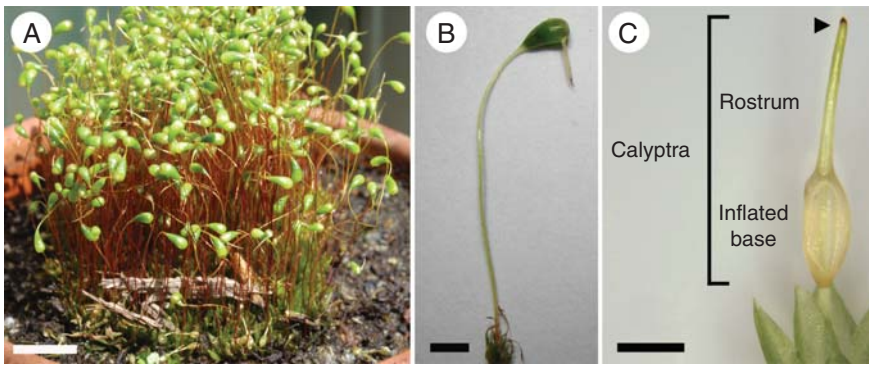

FIG. 1. *Funaria hygrometrica*: (A) population of gametophytes with attached and expanded sporophytes; (B) a mature sporophyte topped by a calyptra; (C) immature, spear-shaped sporophyte covered by a calyptra – the calyptra is divided into a narrow, apical rostrum and a swollen inflated base that narrows in diameter at the bottom, and the sporophyte apex extends into the upper most portion of the rostrum (arrow), during early developmental stages. Scale bars: (A) = 4 mm; (B) = 2 mm; (C) = 1 mm.

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Resin blocks were polymerized under a UV lamp in an EM AFS (Leica, Vienna, Austria) at 0 °C for 4 d, 23 °C for 3 d and then at room temperature for 3 d.

Tissues were sectioned transversely using an Ultrotome III (LKB Produkter, Stockholm, Sweden) to 100 nm. Calyptrae were cut at both the mid rostrum and mid inflated base regions. Sporangia were sectioned through the apex, middle and neck (apophysis). Leafy gametophytes were sectioned in a mature region that included portions of the stem, leaf lamina and leaf midrib. Sections were placed onto 1 × 2-mm gold-coated copper slot grids with a layer of Formvar.

Immunochemistry

To localize cell wall pectins, the rat monoclonal antibody LM19 (Verhertbruggen *et al.*, 2009) was used with the following modifications and additions made to the above protocol. Samples were fixed in 0·25 % glutaraldehyde, 4 % formaldehyde in 0·05 M PIPES buffer (pH 7·0) but without osmium fixation, and tissues were embedded in LRWR. Sections were adhered to 400-mesh gold-coated nickel grids using a Coat Quick 'G' grid coating-pen (Electron Microscopy Sciences, Hatfield, PA, USA) and then dried in a desiccator for a minimum of 14 d. Sections were pretreated with an aldehyde scavenger (37·5 mg glycine, 10 mg sodium borohydride) in 10 mL Tris-buffered saline (TBS), then 1 % normal goat serum (NGS) in TBS, followed by 2 % NGS with 0·1 % fish skin gelatin in TBS. Samples were then incubated in a 1 : 9 dilution of the primary antibody LM19 for 23 h, followed by a 1 : 25 dilution of the goat anti-rat IgG antibody linked to 12 nm colloidal gold for 2 h. Finally samples were rinsed once in TBS, followed by two rinses with water.

All grids were dried and then stained using combinations of separate 1·5 % potassium permanganate ($KMnO_4$), 2 % aqueous uranyl acetate and a 2·5 % lead citrate solutions. Sections were examined and photographed using either an EM300 (Philips Electronic Instruments, Mahwah, NJ, USA) or Tecnai Biotwin (FEI Electron Optics, Eindhoven, the Netherlands) transmission electron microscope at 80 kV. All images were measured using ImageJ (National Institute of Health, Bethesda, MD, USA) for three equally spaced epidermal cells in each tissue region and averaged. The mean value for each region was calculated for all individuals examined. Data analyses (*t*-test and ANOVA) were carried out and graphical figures were created using R 2·11·0 (R Development Core Team, 2010). Prior to *t*-test calculations, variances in each of the groups were compared (Bartlett test of homogeneity of variances in R).

RESULTS

Surface morphology

All gametophyte tissues have a granular ornamentation that is removed by chloroform, but not water rinses. This ornamentation ranges from 0·5 to 1·0 μm in diameter, with a lower density of ornamentation on the rostrum (Fig. 2A) than on the inflated base (Fig. 2D). On the leafy gametophyte tissues (including stem and leaf midrib, not shown), an equally sized granular ornamentation is arranged in rows (Fig. 2G). The sporophyte ornamentation consists of narrow ridges 0·5 μm tall and up to 5 μm long (Fig. 2J). Exterior ornamentation on all tissues was severely altered or completely rinsed away by chloroform washes (Fig. 2B, E, H, K), indicating a chemical composition of lipids or waxes. Small divets remain in the underlying layer where the granular ornamentation was previously observed (Fig. 2B, E, H).

Cuticle anatomy

The cuticle comprises visually distinct layers that were identified and defined following Jeffree (2006) (Fig. 3; modified from Bargel *et al.*, 2006). The location of the exterior cell wall edge was confirmed by labelling the homogalacturonan polysaccharides, a major component of cell wall pectins, with the antibody LM19 (Fig. 2I, L; Verhertbruggen *et al.*, 2009). The cuticular layer (CL) appears as a dark region of the outermost edge of the cell wall (Figs 3 and 4B). Distinct interior and exterior regions of the CL were not observed. Cell wall projections (CWPs) are observed as a texture on the outside edge of the cell wall (part of the CL; Fig. 4B). Exterior to these projections lies the cuticle proper (CP; Figs 3 and 4B). Both an electron-lucent and an electron-dense layer were observed in the CP. The electron-lucent CP appears as a uniform light grey layer just exterior to the dark edge of the cell wall matrix (Figs 3 and 4B). Cell wall material was neither detected visually (Fig. 5) nor with the LM19 antibodies (Fig. 2I, L) in the electron-lucent region, and thus this region was interpreted as CP, not as part of the exterior CL. The electron-dense CP has a dense line on both the exterior and the interior edges with the area between these edges composed of textured reticulations (Fig. 4B). However, in many samples the exterior edge is absent. When stained with $KMnO_4$, a stain specific for fatty acids, there was no increase in the intensity of the electron-dense CP compared with other layers and the cell wall (data not shown). Between the electron-lucent and electron-dense layers of the CP is a region that lacks electron density and appears void of any material. This is interpreted as a separation of the layers due to specimen processing, based on the large variation in the thickness of this space both within and among samples (indicated by asterisks in Fig. 4). Under TEM, light grey spheres were observed on the exterior edge of the tissue (Fig. 2C, F). Based on their textural uniformity with no interior structures, they are interpreted as lipid globules. These lipid globules are partially embedded in the electron-dense CP with this layer closely surrounding and covering the bases of the lipid globules (Fig. 2C, F).

In addition to gross morphological differences, the rostrum and inflated base of the calyptra (Fig. 1C) differ in the thickness of their cuticles (Fig. 5A–F; cuticle thickness indicated by arrows minus the space indicated by an asterisk). These two regions differ significantly in the thickness of several individual cuticle layers and the number of cells in the circumference (Table 1). The narrow rostrum had a thicker cuticle, composed of thicker individual layers, whereas the inflated base contained 4·5× more cells in circumference (Table 1).

The maternal gametophyte tissues of the calyptra were compared with multiple regions of both the leafy gametophyte [i.e. stem (Fig. 5G–I), leaf lamina and midrib] and sporophyte [i.e. apex, mid capsule (Fig. 5J–L) and neck] to determine

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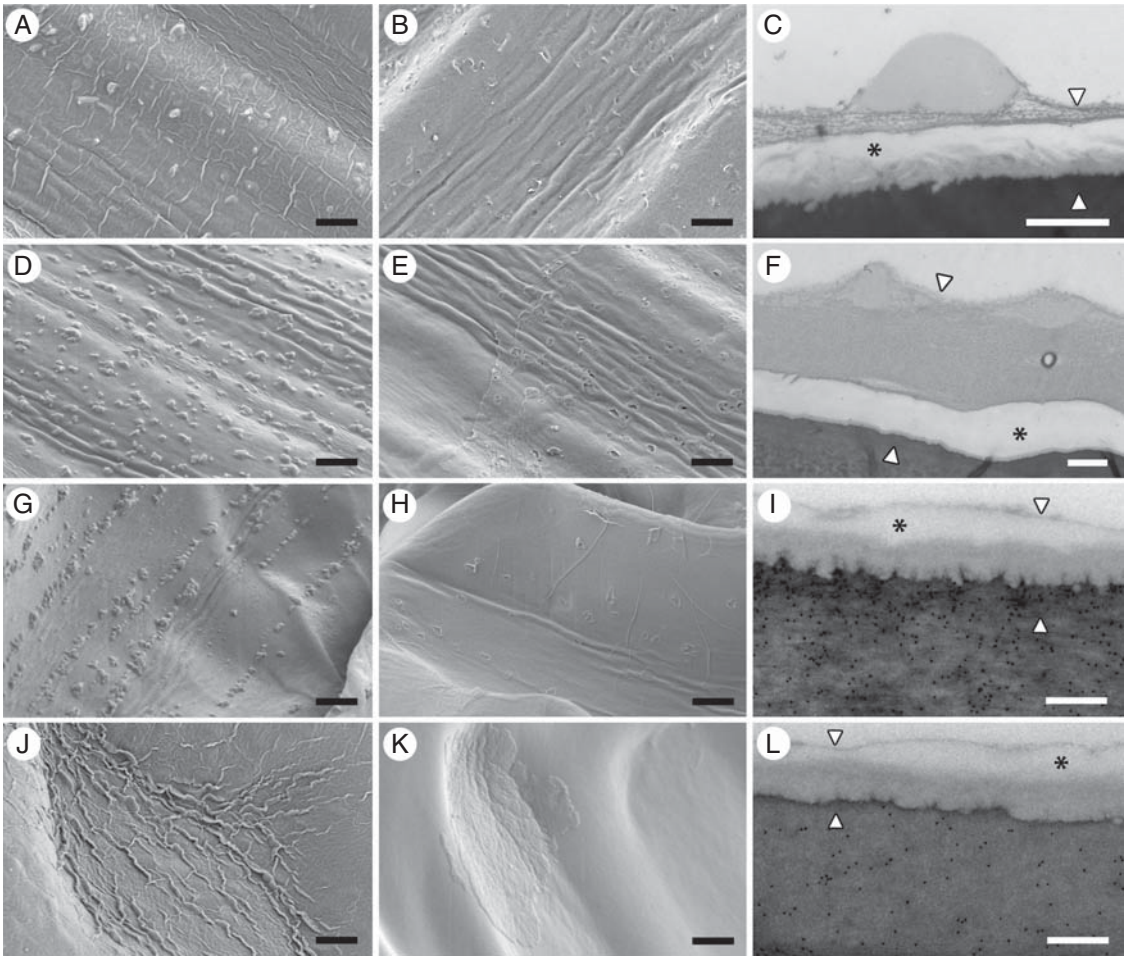

FIG. 2. *Funaria hygrometrica* organs. (A, B, D, E, G, H, J, K) Scanning electron micrographs of the surface morphology: (A, B) calyptra rostrum; (D, E) calyptra inflated base; (G, H) gametophyte leaf lamina; (J, K) sporophyte, mid capsule; (A, D, G, J) air-dried only; (B, E, H, K) air-dried then chloroform rinsed. (C, F, I, L) Transmission electron micrographs of the calyptra rostrum, illustrating the wide variation in thickness of the electron-dense cuticle proper, cuticle thickness indicated by arrowheads minus the space indicated by an asterisk: (C, F) thick electron-dense cuticle proper, with exterior ornamentation of lipid globules; (I, L) thin and faint electron dense cuticle proper – tissues labelled with LM19, specific for cell wall pectin; (I) periclinal cell wall, exterior edge; (L) anticlinal cell wall, exterior edge. Scale bars: (A, B, D, E, G, H, J, K) = 2 μm; (C, F, I, L) = 250 nm.

whether the cuticle of the calyptra was unique relative to other moss organs. All regions of the gametophyte and both the mid capsule and neck of the sporophyte were found to have all four cuticle layers [electron-dense CP (eDCP), electron-lucent CP (eLCP), CWP, CL; Figs 5 and 6]. By contrast, the sporophyte apex lacks both an electron-lucent CP and CWP (J. Budke, unpubl. res.). No statistically significant differences were found between the regions of the leafy gametophyte and sporophyte. Thus, for each leafy gametophyte and sporophyte individual the values for the distinct regions were combined (i.e. mean of stem, leaf midrib and lamina; mean of apex, mid capsule and neck) and used in the subsequent analyses (including Fig. 6). Most cuticle layers on the calyptra were significantly thicker than those of the leafy gametophyte and sporophyte (Fig. 6; ANOVAs comparing the rostrum $n = 3$, inflated base $n = 3$, leafy gametophyte $n = 3$, sporophyte $n = 3$: eDCP, $F_{1,10} = 4{\cdot}03$, $P = 0{\cdot}073$; eLCP, $F_{1,10} = 35{\cdot}56$, $P < 0{\cdot}001$; CWP, $F_{1,10} = 16{\cdot}18$, $P < 0{\cdot}01$; CL, $F_{1,10} = 18{\cdot}53$, $P < 0{\cdot}01$; eDCP + eLCP + CL, $F_{1,10} = 7{\cdot}69$, $P = 0{\cdot}020$).

On the calyptra rostrum a significantly thicker CL was found on the anticlinal cell walls (Fig. 5C; anticlinal 905·88 ± 64·21 nm, periclinal 168·59 ± 11·71 nm, paired *t*-test, var.equal = true, $t = -13{\cdot}99$, df = 2, $P < 0{\cdot}01$). This difference in CL thickness, termed cuticular pegs, was observed on all sections of the rostrum and was completely absent from the inflated base of the calyptra. No differences in the cuticle layers were observed between the anticlinal and periclinal cell walls for either the leafy gametophyte or sporophyte (Fig. 5G–L; data not shown).

DISCUSSION

By applying a system of vascular plant terminology to a bryophyte, we are endeavouring to operate within current cuticle nomenclature and to distinguish anatomically equivalent layers that may be homologous to those of other plants. Cuticle terminology has undergone perpetual revision over the past 50 years (Holloway, 1982). Here we have applied the most recent system of Jeffree (2006). Further chemical

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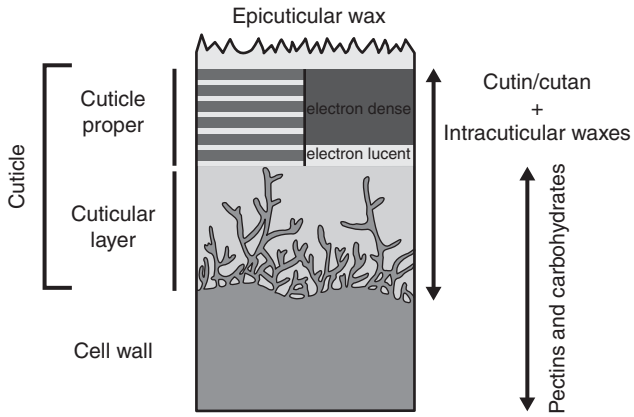

FIG. 3. Diagram illustrating layers and composition of the cuticle, modified from Bargel *et al.* (2006). Pectins and carbohydrates compose the cell wall. The exterior-most edge of the cell wall that intermingles with cutin and waxes is the cuticular layer. This layer is part of both the cell wall and the cuticle. The cuticle proper is a layering of cutins and waxes that are exterior to the cell wall. In vascular plants, multiple layers of electron-lucent and electron-dense lamellae are present (left half of the cuticle proper). For the bryophyte examined herein only a single electron-lucent and single electron-dense layer was observed (right half of the cuticle proper). Exterior to the cuticle proper is a layer of epicuticular waxes that forms a variety of ornamentations.

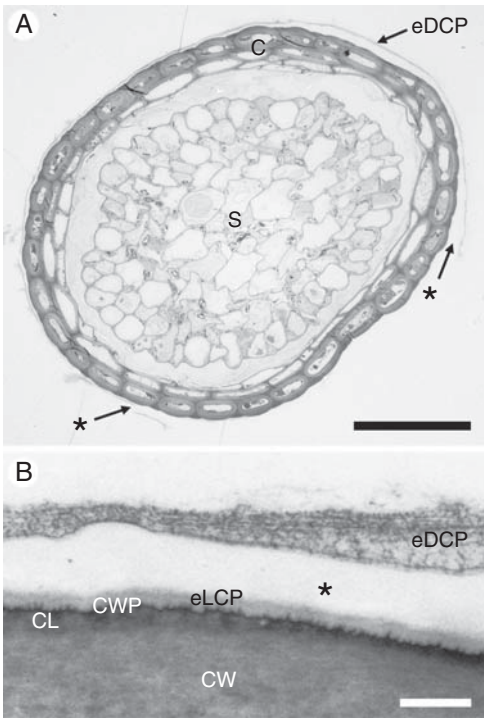

FIG. 4. Transmission electron micrographs of *Funaria hygrometrica*: (A) transverse section through the rostrum region of the calyptra with the sporophyte tissue internally – the outermost layer of the cuticle is separated from the layer beneath and on some sides is completely lacking; (B) outer edge of the calyptra cell wall with the layers of the cuticle identified. Abbreviations: C, calyptra; CL, cuticular layer; CW, cell wall; CWP, cell wall projections of the cuticular layer; eDCP, electron-dense cuticle proper; eLCP, electron-lucent cuticle proper; S, sporophyte. An asterisk (*) indicates a separation between the cuticle layers. Scale bars: (A) = 20 μm; (B) = 500 nm.

and anatomical analyses will be needed from all three bryophyte lineages to make definitive homology assessments relative to other plants. Based on our data, the structure we recognize as the cuticle is present on all organs of the moss body in *F. hygrometrica* and is multi-layered and anatomically similar to the CL, CWPs, eLCP and eDCP of vascular plants. Using different fixation and embedding protocols, Sack and Paolillo (1983) observed both a CL and CWP on the sporophytes of *F. hygrometrica* similar to those reported here. Several bryophytes and algae examined by Cook and Graham (1998) contained an osmophilic and amorphous layer that was identified on the cell exterior as a 'cuticle-like layer' equivalent to the procuticle in vascular plants, most closely resembling the CL in *F. hygrometrica.* Additionally, vascular plants and bryophytes, including *F. hygrometrica*, develop an amorphous eLCP (Jeffree, 2006, figs 2·4c and 2·10a–c).

The main difference between the cuticles of vascular plants and *F. hygrometrica* is the layering of the CP. Typically in vascular plants multiple stacked layers of eDCP, each 2–5 μm thick, alternate with the eLCP layers (Fig. 3, left half; Jeffree, 2006). This arrangement was observed only on the sporophyte guard cells of *F. hygrometrica* (Sack and Paolillo, 1983). In all other cells of *F. hygrometrica* the eDCP is composed of a single electron-dense layer (Figs 3, right half, and 4B) that is much thicker on the calyptra than other tissues (Figs 2F, 5 and 6). The eDCP often lacked an exterior edge and the physical loss of this layer's contents, due to specimen processing, resulted in a decreased density within the layer (Fig. 2C, I, L). Both the complete loss of this layer in addition to separation between the eDCP and eLCP layers were observed (Fig. 4). These artefacts of specimen processing occurred on all organs, more often occurring on organs with a thick eDCP (Fig. 5). The amount of eDCP loss and separation between the layers varied around the circumference of an individual section (Fig. 4A). Despite the elimination of some relatively harsh chemicals used in TEM processing and polymerization of the resin at low (0 °C) temperature, these artefacts persisted. The sensitivity of the eDCP to physical and chemical extraction in *F. hygrometrica* may indicate an increased content of soluble cuticular lipids.

Externally the cuticle bears two distinct types of ornamentation (i.e. granular and sclariform; Fig. 2A, D, G, J). Based on results of the chloroform rinses (Fig. 2B, E, H, K) and the anatomical observations from TEM (Fig. 2C, F), the exterior ornamentation is most likely composed of lipids. These lipid globules do not appear to have a bounding membrane, which would be anticipated in an aqueous environment (Gunning and Steer, 1996). Proctor (1979) also examined *F. hygrometrica* and observed 'trace' ornamentation on the leafy gametophytes, which is most likely equivalent to the granular ornamentation we observed (Fig. 2A, D, G).

The cuticle of the calyptra is thicker than the cuticle of either the leafy gametophyte or sporophyte of *F. hygrometrica* (Figs 5 and 6). Within the calyptra, the cuticle on the rostrum is significantly thicker than that covering the inflated base (Figs 5A–F and 6; Table 1), indicating a functional difference between the two regions. During early sporophyte development, the sporophyte apex, which includes the undifferentiated sporangium and the actively dividing seta meristem (French and Paolillo,

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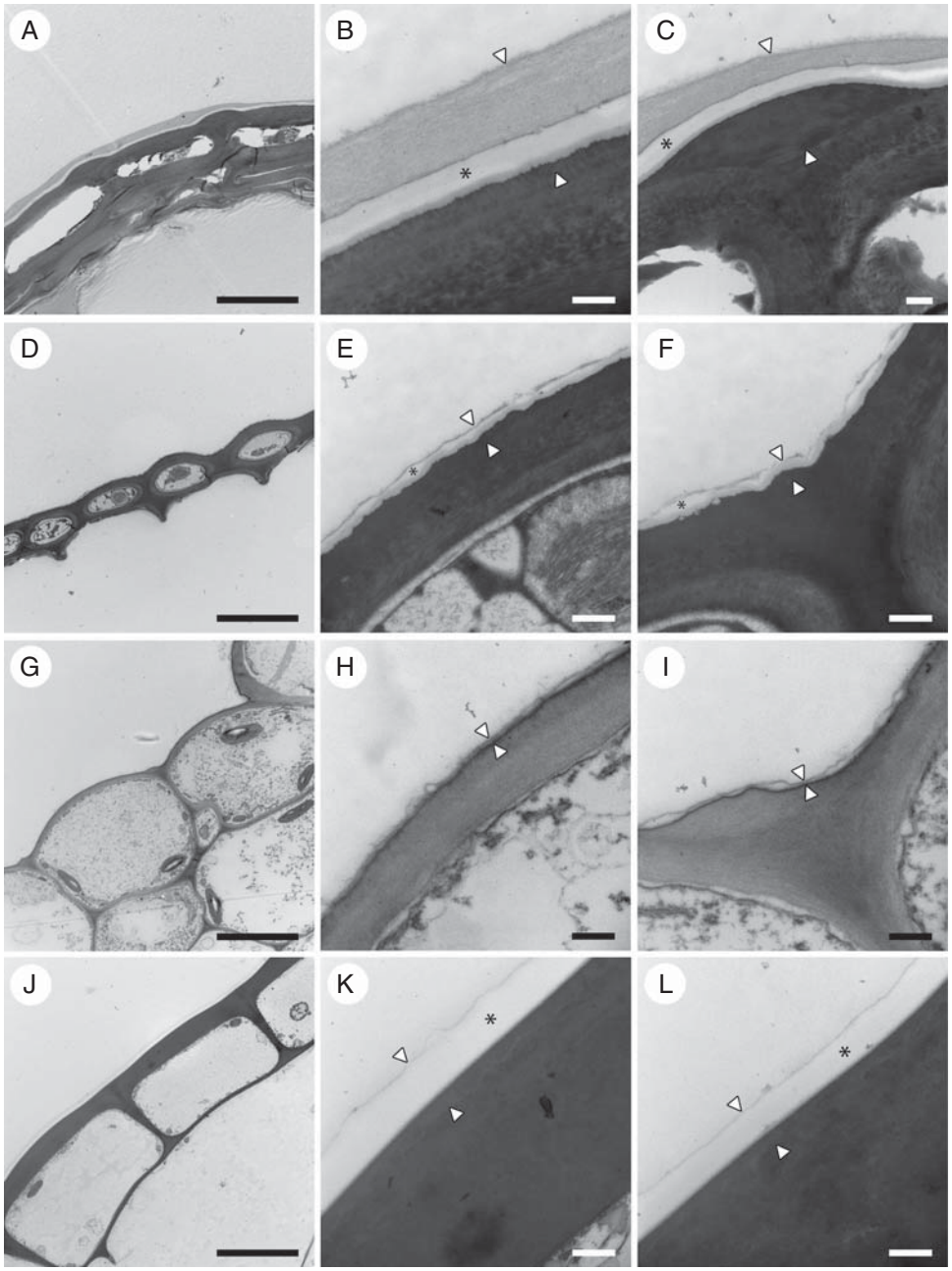

FIG. 5. Transmission electron micrographs of *Funaria hygrometrica* at the edge of the exterior-most cell: (A–C) calyptra rostrum; (D–F) calyptra inflated base; (G–I) leafy gametophyte stem; (J–L) sporophyte, mid capsule; (B, E, H, K) exterior edge of periclinal cell wall; (C, F, I, L) exterior edge of anticlinal cell wall. Cuticle thickness is indicated by arrowheads minus the space indicated by an asterisk. Scale bars: (A, D, G, J) = 10 μm; (B, C, E, F, H, I, K, L) = 500 nm.

1975*b*), is completely nested inside the rostrum (Fig. 1C). At this developmental stage, the inflated base exclusively covers mature portions of the seta below. The thicker cuticle on the rostrum may be functioning to prevent desiccation of these zones that are integral to spore and seta formation. In studies of vascular plants, cuticle thickness and waterproofing ability are uncorrelated (Riederer and Schreiber, 2001); however, this remains untested in bryophytes.

Another cuticle feature, unique to the rostrum of *F. hygrometrica*, is a thickening of the CL at the region of the anticlinal cell walls (Fig. 5C). These cuticular pegs have been found in various seed plants (Norris, 1974; Lendzian *et al.*, 1986), and have been observed in the hornwort *Notothylas* (Cook and Graham, 1998); they are reported here for the first time in a moss. The cells of the calyptra remain alive, with an intact cell membrane, until long after detaching from the maternal gametophyte (Bopp, 1954; Oehlkers and Bopp, 1957); thus, the apoplastic pathways through the calyptra anticlinal cell walls present an unregulated route for water loss from the underlying sporophyte apex. Relative to the calyptra rostrum, the sporangium has a thin cuticle late in development (Fig. 5J–L) and an even thinner cuticle at

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TABLE 1. *Rostrum (*n = *3) and inflated base (*n = *3) anatomy of fully developed* Funaria hygrometrica *calyptra*

Character	Rostrum (nm)	Inflated base (nm)	*t*-test
Electron-dense cuticle proper (eDCP)	496·44 ± 337·95	97·29 ± 67·91	$t = 1{\cdot}48$, df = 2, $P = 0{\cdot}278$
Electron-lucent cuticle proper (eLCP)	89·09 ± 5·79	50·25 ± 6·94	$t = 29{\cdot}53$, df = 2, $\mathbf{P < 0{\cdot}01}$
Cell wall projections of the cuticular layer (CWP)	21·02 ± 3·23	4·34 ± 1·83	$t = 7{\cdot}27$, df = 2, $\mathbf{P < 0{\cdot}05}$
Cuticular layer (CL)	168·59 ± 11·71	101·78 ± 8·08	$t = 5{\cdot}80$, df = 2, $\mathbf{P < 0{\cdot}05}$
Sum of eDCP, eLCP and CL	754·12 ± 346·28	249·32 ± 64·63	$t = 1{\cdot}79$, df = 2, $P = 0{\cdot}216$
Number of cells in circumference	32·33 ± 3·67	145·67 ± 17·80	$t = -5{\cdot}91$, df = 2, $\mathbf{P < 0{\cdot}05}$

For each individual, cuticle layer thicknesses were measured in regions of the periclinal cell wall at three separate epidermal locations around the circumference and then averaged (grand mean ± s.e.). Variances were not significantly different, thus paired *t*-tests were calculated with var.equal = true. Statistically significant tests are highlighted in bold.

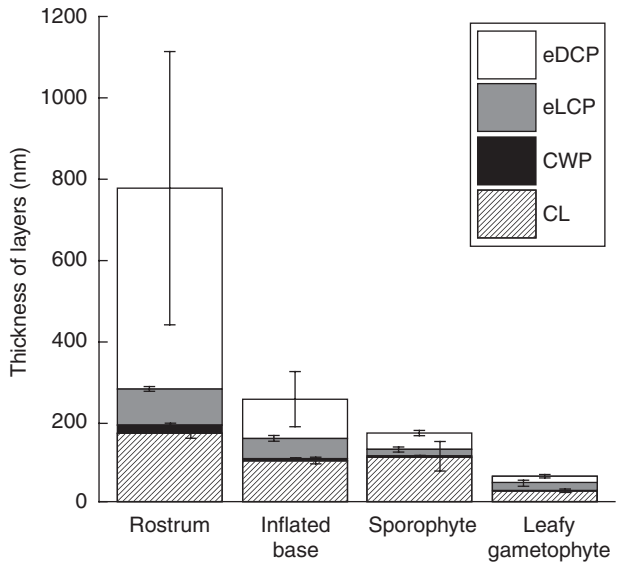

FIG. 6. Thickness of layers measured from *Funaria hygrometrica* for three individuals each for the rostrum, inflated base, sporophyte and leafy gametophyte. Cumulative bar graph with grand mean thicknesses of each layer with ±1 standard error bars. Abbreviations: CL, cuticular layer; CWP, cell wall projections of the cuticular layer; eDCP, electron-dense cuticle proper; eLCP, electron-lucent cuticle proper.

earlier developmental stages (J. Budke, unpubl. res.) and therefore may be unable to protect itself from desiccation. Removal of the calyptra increases the rate of water loss from the sporophyte (Bopp and Stehle, 1957). Thus, the thick calyptra cuticle most likely contributes to the decreased water loss from the immature sporophyte with the cuticular pegs specifically protecting the apoplastic spaces. These anatomical observations support the calyptra rostrum having a unique waterproofing function relative to the inflated base and other moss organs. Our observations of a specialized calyptra cuticle support the hypothesis that this maternal gametophytic organ functions to prevent desiccation of the immature sporophyte apex. This function is analogous to the maternally produced cuticle on the seeds or fruits of angiosperms that protects them from desiccation (Esau, 1977). This is the first demonstration of maternal care via a cuticle in bryophytes.

Two of the earliest diverging moss lineages (i.e. Sphagnopsida and Andreaeopsida) do not share the typical calyptra development that is present in all other mosses (Renzaglia *et al.*, 1997), including *F. hygrometrica.* Their development more closely resembles liverworts, with the surrounding gametophyte tissue remaining intact during early sporophyte development and only rupturing after meiosis, resulting in either a tattered (Duckett *et al.*, 2009, fig. 3b, c) or stunted (Murray, 1988, figs 6 : 1, 7 : 1) calyptra. The transition to a calyptra that detaches from the remainder of the maternal gametophyte in early sporophyte development may have been a critical innovation during moss sporophyte evolution that permitted the elaboration of features, such as stomata and peristome teeth. The protection provided by the calyptra and its associated cuticle may have favoured a taller sporophyte stalk via a seta meristem and increased architectural complexity of the capsule. Calyptrae themselves also vary widely in shape, size and surface projections across genera (Schofield and Hébant, 1984), adding to the complexity of the calyptra–sporophyte interaction in mosses. A broader survey of moss species will be necessary to examine more thoroughly the role of the calyptra in sporophyte development and evolution.

The presence of structurally analogous cuticles in bryophytes suggests an early evolution of this feature in embryophytes (Cook and Graham, 1998). Assessing the homology between bryophyte and vascular plant cuticles will necessitate both chemical and structural analyses, perhaps most importantly in the early diverging bryophyte lineages (i.e. Haplomitriopsida, Takakiopsida, Sphagnopsida, Leiosporocerotopsida). The cuticle most probably developed a waterproofing role on gametophyte tissues prior to its extensive elaboration on sporophyte tissues of vascular plants. The *F. hygrometrica* calyptra has a multi-layered cuticle and cuticular pegs, which in mosses are unique to this organ, thus representing one of the most complex cuticles documented in bryophytes. With these features the calyptra has the potential to play a critical role in desiccation prevention of the developing sporophyte. Experimental studies using calyptra manipulations under different desiccation regimes may garner additional evidence to support the waterproofing function of the moss calyptra.

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